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(54) Title: POLYUNSATURATED FATTY ACYL-PEPTIDE COMPOSITION


(57) Abstract

A fatty acyl-peptide composition useful as an inhibitor of cell proliferation is disclosed. The composition includes a polyunsaturated fatty acyl moiety covalently attached to a peptide. Also disclosed is a method of enhancing anti-proliferative activity of a peptide, by covalently linking the peptide to a polyunsaturated fatty acid.

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# POLYUNSATURATED FATTY ACYL-PEPTIDE COMPOSITION

## Field of the Invention

The present invention relates to polyunsaturated fatty acyl-peptide composition useful in inhibiting cell proliferation.

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## Background of the Invention

Uncontrolled cell proliferation is a characteristic of a number of diseased states. Such growth is observed, for example, in benign and malignant tumors, certain virally-induced diseases and psoriasis. Generally, drugs used to treat cellular abnormalities characterized by uncontrolled cell growth target important biochemical steps or processes that are part of the cell growth cycle. However, such drugs lack selectivity and inhibit the growth of both diseased and healthy cells. Therefore, development of chemotherapeutic agents having relatively high selectivity for the diseased cells would be advantageous.

Agents based on peptide hormones, steroid hormones, hormone-releasing factors, and their respective antagonists and agonists are relatively specific to their target cells. Proliferative cells, such as neoplastic cells or tumors, which arise from hormone-sensitive tissues generally are found to have hormonal requirements that are similar to those of their healthy counterparts. By altering the amount of hormone in the blood circulation it may be possible to selectively restrict the growth of these cells. However, due to the relatively high levels of hormone required for such treatment, use of hormonal chemotherapeutic agents is still limited *in vivo* by toxic side effects to normal cells.

Likewise, cell proliferation may also be inhibited by targeting one or more of the cellular signal transduction systems implicated in the regulation of cell division. These include a) the tyrosine kinase signal transduction

pathway, b) the phospholipid metabolism/protein kinase C signal transduction pathway, and c) the cAMP protein kinase A signal transduction pathway. These pathways are activated by endogenous ligands which initiate a cascade of signalling events that eventually results in cell division. Protein kinases have been found to be particularly important regulators of these pathways. For example, tyrosine kinases are known to play a critical role in the regulation of cell division. High levels of tyrosine kinase activity have been measured in highly proliferative cells, such as neoplastic cells. Inhibition of such phosphorylation activity can be correlated with a reduction in cell division, in some cases.

The current invention is directed to fatty acyl-peptide compositions having enhanced biological activity, compared to the peptide alone. Peptides used to form compositions directed to the inhibition of cell proliferation include peptide hormones, peptide hormone analogues, and protein kinase peptide substrates or peptide inhibitors. In experiments in support of the current invention, it has been found that linking such peptides to the polyunsaturated fatty acids lowers the concentration at least several-fold of such peptides required to inhibit cell proliferation. Such low chemotherapeutic drug concentrations confer the advantage of reducing toxicity to healthy cells.

**Summary of the Invention**

One general object of the invention is to provide a fatty acyl-peptide composition which is useful in inhibiting cell proliferation, such as neoplastic cell proliferation. The composition includes a peptide having antiproliferative activity and conjugated to the peptide, a polyunsaturated fatty acyl moiety. The composition is characterized by a cell proliferative inhibitory activity

which is several-fold greater than that of the antiproliferative peptide alone.

In one embodiment the fatty acyl moiety of the composition is a docosahexaenoyl or an eicosapentaenoyl moiety. In a preferred embodiment, the fatty acyl moiety is a cis-4,7,10,13,16,19-docosahexaenoyl (DHA) or cis-5,8,11,14,17-eicosapentaenoyl (EPA) moiety. The fatty acyl moiety is preferably conjugated to the peptide through an amide linkage.

In another embodiment, the peptide portion of the composition is a peptide hormone, and in a preferred embodiment, the peptide hormone is a somatostatin analog or a gonadotropin releasing hormone (GnRH) analog. In yet another preferred embodiment, the peptide hormone has the sequence of SEQ ID NO: 4, and in another preferred embodiment, the peptide hormone of the composition has the peptide sequence of SEQ ID NO: 5.

The peptide used in forming the fatty acyl-peptide antiproliferative composition can also be a protein kinase modulatory peptide. In a preferred embodiment, the protein kinase modulatory peptide has a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 6.

Another general object of the invention is to provide a method for producing enhanced biological activity of a peptide. In one aspect, the invention is used for producing a several-fold enhancement of cell proliferative inhibitory activity in a peptide composition. According to the invention, this enhancement is achieved by conjugating the peptide to a polyunsaturated fatty acid. Preferred peptides and fatty acyl moieties in the method are described above.

In a more general aspect, the invention provides fatty acyl-peptide compositions having enhanced biological activity, compared to the activity of the peptide alone. Such enhanced biological activity includes such activities

as enhanced hormone activity, anti-tumor activity, enhanced immunogenic activity and other peptide-specific activities.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

**Brief Description of the Figures**

Figure 1 shows exemplary peptide sequences, designated sequences 1-31 and identified by SEQ ID NO: 1-31, respectively, used in forming the novel peptide-fatty acyl compositions of the invention: STKS (sequence 1, SEQ. ID NO: 1), STKSI (sequence 2, SEQ ID NO: 2), SPKCS (sequence 3, SEQ. ID NO: 3), somatostatin analogue (sequence 4, SEQ. ID NO: 4 ), where lower case "d" signifies the presence of a D-amino acid residue and "$NH_2$" at the C-terminal signifies the amidation of the C-terminal end, GnRH (sequence 5, DHA-SEQ. ID NO: 5), where "Glp" signifies the presence of pyroglutamate, EGFA (sequence 6, SEQ ID NO: 6); an N-terminal sequence of human PTH (sequence 7, SEQ ID NO: 7), a polypeptide fragment derived from fibronectin (sequence 8, SEQ ID NO: 8), and a T cell epitope modulatory peptide (sequence 9, SEQ ID NO: 9) suitable for use in forming compositions of the present invention; the sequences of protein kinase modulatory peptides, such as tyrosine kinase inhibitory peptides (sequence 10, SEQ ID NO: 10), (sequence 11, SEQ ID NO: 11), (sequence 12, SEQ ID NO: 12) where "ACM" signifies that the cysteine sulfhydryl group has been modified by an acetamidomethyl group, (sequence 13, SEQ ID NO: 13) and (sequence 14, SEQ ID NO: 14), calmodulin-dependent protein kinase III inhibitory peptide (sequence 15, SEQ ID NO: 15), dsDNA-dependent kinase inhibitory peptide (sequence 16, SEQ ID NO: 16), protein kinase C modulatory peptide (sequence 17, SEQ ID NO: 17), and other protein kinase modulatory peptides, such as for cAMP dependent kinase (sequence 18, SEQ

ID NO:18), (sequence 19, SEQ ID NO: 19), and (sequence 20, SEQ ID NO: 20), phosphorylase kinase (sequence 21, SEQ ID NO: 21), calmodulin-dependent kinase I and II (sequence 22, SEQ ID NO: 22 and sequence 23, SEQ ID NO: 23), dsRNA-dependent kinase (sequence 24, SEQ ID NO: 24), proline-dependent kinase (sequence 25, SEQ ID NO: 25 and sequence 26, SEQ ID NO:26), growth factor-regulated kinase (sequence 27, SEQ ID NO: 27), casein kinase I and II (sequence 28, SEQ ID NO:28 and sequence 29, SEQ ID NO: 29, AMP-activated protein kinase (sequence 30, SEQ ID NO:30), and S6 kinase II (sequence 31, SEQ ID NO:31);

Figure 2 illustrates coupling of cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) to a peptide (sequence 1, SEQ ID NO: 1) through an N-terminal amine group to form the fatty acyl-peptide composition (composition I, DHA-SEQ ID NO: 1); and

Figure 3 shows sequences of exemplary fatty acyl-peptide compositions of the invention: DHA-STKS (composition I, DHA-SEQ. ID NO.: 1), DHA-STKSI (composition II, DHA-SEQ ID NO: 2), DHA-SPKCS (composition III, DHA-SEQ. ID NO.: 3), DHA-somatostatin analogue (composition IV, DHA-SEQ. ID NO.: 4 ), D-Lys6(DHA)-GnRH (composition V, DHA-SEQ. ID NO.: 5), and DHA-EGFA (composition VI, DHA-SEQ ID NO:6), where DHA is a cis-4,7,10,13,16,19-docosahexaenoyl moiety, Glp represents pyroglutamate, and lower case "d" signifies the presence of a D-amino acid residue, and "$NH_2$" at the C-terminal signifies the amidation of the C-terminal end.

**Detailed Description of the Invention**

I. Definitions

The term "polyunsaturated fatty acid" refers to a compound having a carboxylic acid moiety and a long unbranched carbon chain, usually containing between about 8 and 24 carbon atoms, and containing two or more carbon-carbon double bonds. When a fatty acid is conjugated to a peptide through an amide linkage, a fatty acyl-peptide derivative is formed. The disclosed invention includes fatty acyl-

peptide derivatives, and, more generally, peptides linked to long unbranched, polyunsaturated carbon chains.

The term "peptide hormone" refers to a peptide that elicits a biological response in a target cell. Peptide hormones are generally low molecular weight proteins (< 50,000). Such hormones can be isolated from biological sources, chemically synthesized or produced by recombinant methods. Generally, in their natural setting, peptide hormones are secreted from specific cells and produce biological effects in other cells. Analogues of such naturally occurring hormones are encompassed by the term "peptide hormone" and are produced recombinantly or synthetically.

Peptide hormones, as described herein, are divided into two main categories, according to their known activities *in vivo*. Generally, hormones act either directly or indirectly to produce the desired biological effect. In the context of the present invention, the desired effect is inhibition of cell proliferation. Peptide hormones that act directly on a cell to inhibit division of the cell are referred to herein as direct peptide hormones; those peptides that act to stimulate or inhibit synthesis or secretion of endogenous peptide growth regulatory hormones are referred to as indirect peptide hormones.

Generally, it will be seen that an indirect peptide hormone effects a change (increase or decrease) in the extracellular levels of a naturally occurring direct peptide hormone. Two exemplary types of indirect peptide hormones are peptide hormone-releasing hormones and peptide hormone release-inhibiting hormones. Naturally occurring indirect peptide hormones are generally short polypeptides, usually under 20 amino acids in length.

Peptide hormone analogs are synthetically or recombinantly prepared peptides which are structurally similar to naturally occurring peptide hormones. For the purposes of the present invention, such peptide analogs are

included by the term "peptide hormone." Generally, to be useful in the present invention, such analogs have essentially a similar or a higher biological activity than that of the endogenous peptide hormone.

Protein kinase modulatory peptides are peptides which act as inhibitors of protein kinase activity. Such peptides may act, for example, as protein kinase substrates, containing a phosphorylatable amino acid residue in the sequence. Alternatively, such kinase modulatory peptides may bind to the kinase catalytic site, to effect inhibition of kinase activity. Such kinase modulatory peptides act to produce reduced phosphorylation of endogenous protein kinase substrates.

Peptides and compositions that inhibit cell proliferation are sometimes referred to herein as "anti-proliferative" peptides or compositions.

## II. Selection of Peptides for Use in the Fatty acyl-Peptide Composition

### A. Selection of Peptide Hormones

The present invention is directed to peptide compositions having anti-proliferative activity. More generally, it is the discovery of the invention that these fatty acyl-peptide compositions have enhanced biological activity in comparison to underivatized peptides. Anti-proliferative fatty acyl-peptide compositions of the invention are effective to inhibit proliferation of highly proliferative cells, such as neoplastic cells or virally-infected cells. For use in the compositions of the invention, peptides known to have anti-proliferative activity are linked to a fatty acyl moiety, such as a docosahexaenoyl (DHA) or eicosapentaenoyl (EPA) group, as described below. Peptide hormones having cell proliferative inhibitory activity are known in the art, and may be direct or indirect hormones. Such anti-proliferative peptide hormones are usually, but not

necessarily, specific to a particular type of cell, such as a cell with specific hormonal requirements.

1. Indirect Peptide Hormones. As noted above, in the context of the present invention, indirect peptide hormones include those peptides which act to effect a decrease in the level of a direct cell proliferation stimulatory hormone or to effect an increase in the level of a direct cell proliferation inhibitory hormone.

An example of an indirect peptide hormone which stimulates release of an antiproliferative hormone in some mammalian species is gonadotropin releasing hormone (GnRH). GnRH stimulates release of several gonadotropins, including luteinizing hormone (LH). GnRH and GnRH analogues can also inhibit the growth of hormone dependent carcinomas, such as androgen-dependent prostate tumors (Schally). GnRH is also effective against such tumors as colon and pancreatic tumors. Figure 1 shows a sequence of a GnRH analog useful in treating such tumors (5, SEQ ID NO: 5). Figure 3 shows a DHA-GnRH fatty acyl-peptide composition of the invention (V, SEQ ID NO: 5) having enhanced activity, as described below.

GnRH analogues can be screened for potential anti-proliferative activity, by assessing their abilities to stimulate release of LH from anterior pituitary cells, as detailed in Example 9. Active GnRH analogue peptides are then used to form fatty acyl peptide compositions of this invention, and tested for enhanced biological activity, as discussed below.

Indirect peptide hormones also include peptide hormone release-inhibiting hormones. An exemplary peptide hormone of this type is somatostatin. Somatostatin is a 14 amino acid peptide that inhibits the release of growth hormone (GH). GH enhances the proliferation of its cellular targets. Native somatostatin peptide and analogs are used to form fatty acyl peptide derivative compositions of the

invention, as described below. An analog of somatostatin used to form fatty acyl peptide compositions in studies described herein has the sequence 4 (SEQ ID NO: 4) in Figure 1, and a fatty acyl-peptide composition which includes this peptide is shown as composition IV (DHA-SEQ ID NO: 4) in Figure 3. The somatostatin fatty-acyl peptide is about 150 times more potent than the parent molecule.

It can be appreciated that other indirect acting peptide hormones can be used in forming fatty acyl-peptide compositions of the invention. Preferably, peptides are selected based on their known anti-proliferative activity in a cell proliferation assay, described in Section III below. Selected peptides are then conjugated to fatty acyl moieties, and are tested for enhanced potency in such assays. A fatty-acyl peptide derivative composition is a useful anti-tumor or anti-neoplastic cell agent, when it is found to have at least a several-fold increase in potency, in comparison to the unconjugated peptide.

2. Direct Peptide Hormones. Also used in forming compositions of the invention are peptides which are known to have direct antiproliferative effects on cells. An exemplary direct peptide hormone is parathyroid hormone (PTH). This peptide is 84 amino acids in length, and can directly inhibit osteoblast division. Certain bone cancers are characterized by hyperproliferating osteoblasts. Structure-function studies indicate that an N-terminal fragment of PTH is active in inhibiting osteoblast proliferation (Kano). This sequence 7 (SEQ ID NO: 7) is shown in Figure 1.

Another example of a peptide which is defined as a direct peptide hormone for purposes of this invention is a peptide fragment of fibronectin having the sequence SEQ ID NO: 8, shown in Figure 1 as sequence 8. This fragment spans the recognition site within the fibronectin molecule to which cells bind for attachment to fibronectin. This

fibronectin recognition site peptide and analogues thereof are used to inhibit fibronectin-mediated cell attachment and spreading in vitro. They may also be important for regulation of tumor cell proliferation and metastasis in vivo (Kumagai). According to the present invention, fibronectin recognition site peptides are coupled to fatty acyl moieties to form compositions useful in inhibiting tumor cell proliferation and metastasis.

Direct peptide hormones, as defined in the current invention, also encompass peptides derived from immunogenic polypeptides for modulating a response from specific immune cell populations. For example, a T-cell epitope peptide (TCEP) is coupled to a fatty acyl moiety as described herein to form a composition useful in modulating the T-cell immune response. The sequence of TCEP (SEQ ID NO:9) is shown in Figure 1 as sequence 9, and synthesis of a DHA derivative of TCEP is detailed in Example 7.

B. Selection of Protein Kinase Modulatory Peptides

Elevated protein kinase C and tyrosine kinase activities are associated with neoplastic cell proliferation or transformation (Weinstein, Yarden). Inhibition of such kinase activities can be effected by the presence of small peptide fragments which mimic protein substrate phosphorylation sites and/or protein kinase modulatory domains. Such protein kinase modulatory peptides are effective to compete with endogenous protein kinase substrates. Selection of specific protein kinase modulatory peptides for use in forming the fatty acyl-peptide derivatives of the invention is described below.

1. Tyrosine Kinase Substrates and Inhibitors.

Receptors for a number of growth factors, including epidermal growth factor (EGF), insulin growth factor (IGF), and platelet derived growth factor (PDGF), contain tyrosine kinase catalytic domains which phosphorylate specific

intracellular protein substrates, including the receptor itself, in some cases, at tyrosine residues. Cellular transformation by a virus, such as the Rous sarcoma virus, can result from expression of a single viral protein, which functions as a tyrosine kinase.

Tyrosine kinases may also play a role in the uncontrolled growth of keratinocytes which can result in psoriasis or other highly proliferative skin disorders. Keratinocytes possess tyrosine kinase-like growth factor receptors.

High tyrosine kinase activity has been associated with high rates of cellular proliferation (Casneillie, Keri). Inhibition of such kinase activity decreases the rate of cellular proliferation. Several tyrosine kinase modulatory peptides which are effective to inhibit tyrosine kinase activity *in vitro* have been described in the literature, and some are available commercially, for example from Peptides International (Louisville, KY). Figure 1 shows sequences of several exemplary tyrosine kinase modulatory peptides which can be used to form anti-proliferative compositions of the invention, such as STKS (SEQ ID NO: 1) and STKSI (SEQ ID NO: 2), sequences 1 and 2, respectively.

Alternatively, tyrosine kinase modulatory peptides can be identified, based on peptide sequences surrounding phosphorylation sites of endogenous protein kinase substrates in highly proliferating cells. Preferably, cellular sources used for identification of such substrate peptides are proliferating cells of the type to be targeted; however, it is appreciated that tyrosine kinase modulatory peptides derived from one cell source may inhibit a tyrosine kinase derived from a different cell type.

In order to isolate an endogenous protein kinase substrate protein, proliferating cells, such as a lymphoma cell line having a high level of tyrosine kinase activity, are incubated with [$^{32}$P]-phosphate, or a particulate

fraction of the cells is incubated with radiolabelled [gamma $^{32}P$] ATP. Cellular polypeptides are separated by denaturing gel electrophoresis, and the phosphorylated proteins are observed by autoradiography. Phosphoprotein-containing bands are excised from the gel, and the phosphoprotein is eluted from the gel band. The eluted protein is subjected to partial hydrolysis, and the identity of the phosphorylated amino acid determined, according to methods known in the art (Casneillie). In order to determine the peptide sequence in the vicinity of the phosphotyrosine residue(s), phosphoproteins are subjected to peptide fragmentation, such as by proteolysis or chemical means, and phosphotyrosine-containing peptides are sequenced, according to conventional methods (Casneillie). Short peptides (approximately 6-20 amino acids) corresponding to the peptide sequences in the vicinity of the phosphorylation site can be synthesized and tested for inhibition of tyrosine kinase activity.

Tyrosine kinase modulatory peptides can be prepared by substituting for the tyrosine phosphorylation site residue an amino acid residue that cannot be phosphorylated, such as a phenylalanine residue. composition II in Figure 3 (DHA-STKSI) shows an inhibitory fatty acyl-peptide composition, the peptide portion of which has almost complete identity with the peptide portion of composition I (DHA-STKS), except that in composition II a phenylalanine residue has been substituted for a tyrosine residue present in composition I. Both peptides inhibit proliferation of neoplastic cells. Accordingly either peptide can be conjugated to a fatty acyl group, to form an anti-tumor fatty acyl-peptide composition of the invention, as described in Section III, below.

2. Protein Kinase C (PKC) Peptide Substrates.

PKC is a component of the phospholipid metabolism/protein kinase C signal transduction pathway which plays a critical

role in normal cellular growth control. Activation of PKC is mediated by a family of G-protein-modulated receptors. When activated, the cytosolic form of PKC binds to the cytoplasmic face of the plasma membrane. One of the known protein targets of PKC is the EGF receptor. Phosphorylation of EGF receptor by protein kinase C results in a decrease in the affinity of the receptor for EGF and a decrease in EGF receptor-associated tyrosine kinase activity (Berridge). This illustrates the heterologous interactions between signal transduction pathways, specifically between the tyrosine kinase pathway and the phospholipid metabolism/protein kinase C pathway.

Recent results demonstrate that protein kinase C activity inhibition is directly related to the anti-proliferative activity of certain gonadotropin-releasing factor agonists. These agonists are effective in inhibiting DNA synthesis in certain breast cancer cell lines. Both follicin and buserelin, the two tested gonadotropin-releasing factor agonists, inhibit protein kinase C activity and tyrosine kinase activity (Keri). It has also been demonstrated that somatostatin analogues can stimulate a phosphatase activity in tumor cells thereby inhibiting signal transduction and tumor growth (Schally).

The sequences of a number of peptide substrates for PKC are known and are commercially available. Figure 1 lists the sequences of some of the known peptide substrate inhibitors for PKC, such as sequence 3 (SEQ ID NO: 3) and sequence 17 (SEQ ID NO: 17). These peptides are suitable PKC modulatory peptides for use in forming fatty acyl-peptide compositions of the present invention. PKC peptide inhibitors can also be identified from endogenous protein substrates of protein kinase C, using the same general procedures described in part 1, above, for tyrosine kinases.

3. Other Protein Kinases. Modulatory peptides targeting protein kinases other than tyrosine kinase and protein kinase C can also be used to form fatty acyl-peptide anti-tumor compositions of the invention. One such protein kinase target is a casein kinase II found in epidermal keratinocytes. High proliferation rates are related to the hyperphosphorylation of an oncogenic product from a human papillomavirus which contains a casein kinase II phosphorylation consensus sequence (Hashida). This casein kinase II consensus phosphorylation sequence is shown in as sequence 29 in Figure 1 (SEQ ID NO: 29).

Another protein kinase, cyclic AMP-dependent protein kinase (PKA), is a component of the cAMP-dependent protein kinase signal transduction pathway. PKA phosphorylates threonine and serine residues of a protein substrate. The activity of this enzyme is enhanced by cAMP. PKA may regulate growth control. In some animal cells, an increase in cAMP levels activates specific genes involved in growth regulation. In neuroendocrine cells of the hypothalamus, cyclic AMP turns on the gene that encodes the peptide hormone release-inhibiting hormone, somatostatin.

Other target protein kinases, in conjunction with the present invention, are components of signalling cascades involved in cell proliferation. For example, when epidermal growth factor (EGF) binds to the epidermal growth factor receptor (EGFR), a tyrosine kinase, it stimulates activation of myelin basic protein (MBP) kinase, through phosphorylation of MBP kinase. MBP kinase, in turn, activates S6 kinase by a phosphorylation event. The result of activation of this signalling pathway is stimulation of proliferation of EGFR-bearing cells, such as adipocyte cells (Ahn). It can be appreciated that fatty acyl-peptide compositions of the invention using as peptide components, phosphorylation recognition sequence peptides for one or more of EGFR kinase, MBP kinase and S6 kinase will serve to inhibit the signalling cascade of which these kinases are a

part. Sequences of exemplary peptide inhibitors of these kinases are shown in Figure 1 (e.g., sequence 11, SEQ ID NO: 11, to sequence 31, SEQ ID NO:31).

Other examples of protein kinase activities involved in cell proliferation whose activities can be modulated, using fatty-acyl peptide compositions of the present invention, include calmodulin-dependent kinases I and II, cGMP-dependent protein kinase, ds-DNA-dependent protein kinase, proline-dependent kinase, and AMP-activated kinase. Sequences of exemplary peptide modulatory peptides directed to these kinases are listed in Figure 1 (SEQ ID NOs: 18-31).

## III. Preparation of Fatty acyl-peptide Compositions

In accordance with the present invention, it has been observed that conjugation of fatty acyl groups to the above-described peptides enhances the biological activity of the peptides. Enhancement of biological activity is exemplified herein as enhancement of inhibition of cell proliferation, such as neoplastic cell proliferation. In experiments carried out in support of the present invention, exemplary peptide hormones and protein kinase modulatory peptides were selected, as described above, and isolated from natural sources or synthesized, as described below. Subsequently, the peptides were conjugated to fatty acyl moieties, as described below and illustrated in Figure 2 and tested for their activities in cell proliferation assays, as described in Section III, below.

### A. Solid Phase Peptide Synthesis

Peptides shorter than about 30 amino acids in length are conveniently prepared by methods commonly used in solid-phase peptide synthesis, as detailed below (Stewart). Briefly, N-alpha-protected amino acid anhydrides are prepared in crystallized form and used for successive amino acid addition to the peptide N-terminus. At each residue

addition, the growing peptide on a solid support is acid treated to remove the protective group, and washed several times to remove residual acid. The peptide is then reacted with another N-protected amino acid. The amino acid addition reaction may be repeated two or three times to increase the yield of growing peptide chains. After completing the growing peptide chains, the protected peptide resin is treated with liquid hydrofluoric acid to deblock and release the peptides from the support.

B. Conjugation of Fatty Acyl Moieties to Peptides

Preferred fatty acyl moieties for use in the present invention are those with a high degree of unsaturation, and include such fatty acids as cis-5,8,11,14,17-eicosapentaenoic (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA). Such polyunsaturated fatty acids can be prepared synthetically according to standard methods, isolated from the oils of marine fish, or obtained from commercial sources.

Generally, fatty acids are linked to the peptides via the terminal amine group or via internal amine groups, such as the amine group of lysine, in an amide linkage. Figure 2 illustrates a scheme for coupling of sequence 1 to DHA by the terminal amino group of the peptide through an amide linkage to form the fatty acyl-peptide composition I in Fig. 3. DHA is activated by reaction with N-hydroxysuccinimide prior to reaction with an amino-group containing polypeptide. In another embodiment, DHA is coupled by the free amine group of a lysine residue in the sequence to form the fatty acyl composition, such as composition V in Fig. 3.

Examples 2-6 detail preparation of fatty acyl-peptide compositions in which acylation of peptides is carried out using activated ester or acyl chloride derivatives of DHA or EPA. Activated esters useful in preparing compositions of the current invention include DHA-O-benztriazole ester

(DHA-OBT), DHA-O-pentafluorophenyl ester (DHA-Opfp), and DHA-O-succinimidyl ester (DHA-O-N-Succ). The acylation is carried out as detailed in Example 2. Crude products, obtained after acylation, are purified by HPLC. The purity of the final products is characterized by analytical HPLC and TLC data, while the chemical characterization is accomplished by mass spectrometry (MS).

C. Fatty Acyl-Peptide Compositions

From the foregoing discussion, it can be appreciated that peptides can be selected for use in anti-proliferative fatty acyl-peptide compositions of the invention, based on their abilities to inhibit cell proliferation or to inhibit components, such as protein kinase components, of cell proliferative stimulus pathways. According to an important feature of the present invention, conjugating to such a peptide, a polyunsaturated long chain carbon, such as a polyunsaturated fatty acyl moiety described above, enhances the biological activity of the peptide. Specifically, conjugation of a polyunsaturated fatty acyl moiety to an anti-proliferative peptide enhances its ability to inhibit neoplastic cell proliferation. This aspect of the invention will be better appreciated from the discussion below.

As an example, tyrosine kinase modulatory peptides selected as described above are covalently linked to polyunsaturated fatty acids to form compositions which are effective to inhibit proliferation of neoplastic cells. One such composition used in experiments carried out in support of the current invention is DHA-STKS (DHA-SEQ. ID NO. 1) illustrated in Figure 3 (composition I).

Fatty acid-tyrosine kinase inhibitory peptide compositions can also be prepared by substituting for the tyrosine residue an amino acid residue that cannot be phosphorylated. In one such composition, DHA-STKSI (DHA-SEQ ID NO: 2; composition II), the tyrosine residue of tyrosine

kinase substrate DHA-STKS (DHA-SEQ ID NO: 1; composition I) has been substituted by a phenylalanine residue. Both fatty acid-protein kinase peptide substrate and peptide inhibitor compositions inhibit the proliferation of neoplastic cells, as detailed in Example 8 and shown in Table 1. Another such tyrosine kinase inhibitor peptide is DHA-EGFA (DHA-SEQ ID NO: 6, composition VI) also shown in Table 1.

A PKC peptide substrate-fatty acid composition used in the experiments described in Example 8 includes a peptide fragment from myelin basic protein, and has the structure shown in Figure 3 as composition III (DHA-SEQ. ID NO: 3). Additionally, endogenous substrates of PKC may be identified as described above for the tyrosine kinase substrates. The polypeptide fragments that contain PKC phosphorylation sites are then sequenced and used to form the fatty acid peptide compositions of the invention.

IV. Anti-Proliferative Assays

In accordance with the present invention, fatty acyl-peptide derivatives are prepared as described in Section II, above, and inhibition of neoplastic cell proliferation by such compositions is measured, according to one or more standard cell proliferation assays. In one exemplary assay, described in Example 8, inhibition of cell proliferation is measured directly, by measuring the number of surviving cells after exposure of the cells to a test composition, such as a fatty acyl-peptide composition disclosed herein.

Alternatively, an activity which correlates to cell proliferation can be measured to determine indirectly the effect of a test compound on cell proliferation. Thus, activities such as tyrosine kinase activity or release of a growth-promoting hormone, which are known to correlate with neoplastic cell proliferation, are used as indicators of anti-proliferative activity. As exemplified herein,

polyunsaturated fatty acyl-peptide anti-proliferative compositions may exhibit one or more of the following *in vitro* activities: (a) inhibition of cell proliferation, (b) inhibition of tyrosine kinase activity, (c) stimulation of release of a growth-inhibiting hormone, such as luteinizing hormone, and (d) inhibition of release of growth-stimulating hormones, such as inhibition of growth-hormone release. It is appreciated that other *in vitro* assays which can be correlated with uncontrolled cell growth may also serve as assays for selecting and determining the activities of fatty acyl-peptide compositions of the invention. More specifically, such assays can serve to determine the relative potencies of compositions of the invention as anti-proliferative agents.

A. Inhibition of Cell Proliferation

An assay for measuring the inhibitory effects of polyunsaturated fatty acyl-peptide derivatives on cell proliferation is described in detail in Example 8. In experiments carried out in support of the invention, a number of tumor cell lines, including a human prostatic adenocarcinoma cell line, a human breast adenocarcinoma cell line and a human colon adenocarcinoma cell line, were used to test compounds of the invention for effects on cell proliferation. Cells were exposed to the tyrosine kinase synthetic peptide substrate fatty acid derivative DHA-STKS (DHA-SEQ ID NO: 1), the protein kinase C peptide substrate fatty acid derivative DHA-STKCS (DHA-SEQ ID NO:3), or the gonadotropin release hormone fatty acid derivative DHA-D-Lys6-GnRH (SEQ ID NO: 5), as described below.

Solutions of the peptide derivatives were added in culture media to test cells for an incubation period of 2.5 hours. At the end of the test period, cells were centrifuged, the pellet diluted with 1% BSA in saline and viable tumor cell counts were determined by the trypan blue exclusion method. Results of tests using peptide fatty

acid derivatives DHA-STKS (DHA-SEQ. ID NO:1) and DHA-D-lys6-GnRH (DHA-SEQ. ID NO: 5) in various transformed cell lines are shown in Table 1. Cell proliferation was inhibited by at least 50% at a peptide-fatty acid derivative concentration of 10 micrograms/ml of the TK modulatory peptide substrate, the PKC modulatory peptide, and the gonadotropin-releasing hormone analogue, as shown in Table 1.

Table 1
Effect of Fatty Acid-Peptide Compositions on the Inhibition of Cell Proliferation
(% Inhibition)

| Compound | Concentration (ug/ml) | | | |
|---|---|---|---|---|
| | 0.1 | 1 | 10 | 30 |
| DHA-STKS (PC3)* | - | - | 90 | - |
| DHA-STKS (PC3) (liposomal) | - | - | 57 | - |
| DHA-SPKCS (MCF 7) | 5 | 25 | 43 | |
| DHA-D-Lys6-GnRH (MCF 7) | - | 33 | 52 | 61 |
| DHA-D-Lys6-GnRH (HT 29) | - | 18 | 28 | 45 |

*Neoplastic cell types tested are indicated in brackets, and are as follows:
PC 3, human prostatic adenocarcinoma cell line;
MCF 7, human breast adenocarcinoma cell line; and
HT 29: human colon adenocarcinoma cell line.

Additional methods are available for monitoring the viability of cells, including assays based on the differential dye uptake by viable cells in comparison to that taken up by non-viable cells. For example, viable cells take up diacetyl fluorescein and hydrolyze it to fluorescein, to which the cell membrane of live cells is impermeable. Live cells fluoresce green. Nonviable cells may be counter-stained with ethidium bromide and will fluoresce red. These methods may be used in flow cytometric assays, in accordance with protocols known in the art.

B. Stimulation of Luteinizing Hormone (LH) Release

Peptide hormone-releasing hormones increase the extracellular levels of peptide hormones that interact with specific cellular receptors. Compounds, such as peptide hormone-releasing hormones, which stimulate production of LH have also been shown to inhibit cellular proliferation. In accordance with the invention, fatty acyl derivatives of such compounds are effective to inhibit neoplastic cell proliferation at concentrations which are several-fold lower than the concentration of peptide alone required to inhibit such cell proliferation.

Specifically, as shown below, an analog of gonadotropin releasing hormone analog conjugated to the polyunsaturated fatty acid, DHA, (D-Lys$^{6(DHA)}$-GnRH; DHA-SEQ ID NO:5) was tested in an assay of LH release by anterior pituitary cell suspensions as detailed in Example 9. In this assay, luteinizing hormone levels were quantitated by a double antibody radioimmunoassay procedure. As shown in Table 2, the polyunsaturated fatty acid-gonadotropin release hormone analogue (D-Lys6(DHA)-GnRH) was about 5-fold more effective in eliciting the release of luteinizing hormone when compared to equivalent concentrations of GnRH or to the D-Lys6-GnRH analogue lacking the fatty acid moiety. The polyunsaturated fatty acid DHA alone had no effect on luteinizing hormone release at the concentration tested (Table 2). These results further demonstrate the effectiveness of compositions of the invention in enhancing biological activity of their peptide components.

Table 2
Effect of the Fatty Acid-GnRH Analogue Composition on LH Release (% Change[a])

| Compound | Concentration (M) | | |
|---|---|---|---|
| | $10^{-10}$ | $10^{-9}$ | $10^{-8}$ |
| D-Lys6(DHA)-GnRH | 10 | 550 | 900 |
| DHA | - | 0 | - |
| GnRH | 23 | 100 | 220 |
| Lys6-GnRH | 20 | 95 | - |

[a]Compared to the amount of LH released by $10^{-9}$M GnRH

C. Inhibition of Growth Hormone Release

Peptide hormone-release inhibiting hormones decrease the extracellular levels of peptide hormones which interact with specific cellular receptors. Generally, binding of peptide hormone to the receptor triggers a cascade of biochemical events mediated through second messengers. In some cases, an end-result of such hormonal activity is cellular proliferation. Peptide hormone-release inhibiting hormones which act to inhibit such hormones are useful in forming fatty acid-peptide compositions described by the present invention. Analogues of such peptide hormone-release inhibiting hormones are also useful in forming such compositions.

Somatostatin is an exemplary peptide hormone-release inhibiting hormone analogue which inhibits hormonally activated cell proliferation. Somatostatin is a growth hormone release inhibitory hormone which inhibits release of growth hormone. Growth hormone (GH) binds to specific cell-surface receptors distributed widely throughout the body. Binding of GH agonists to GH receptors results in increased cellular division, and hence, cell proliferation.

In experiments carried out in support of the present invention, a somatostatin-fatty acyl analogue (SEQ ID NO:

4) was synthesized as detailed in Example 5. Inhibition of growth hormone release by the DHA-acylated somatostatin analogue was measured, as described in Example 10. GH levels were determined by a double-antibody radioimmunoassay for the hormone. In this assay, the DHA-somatostatin analogue was 1000 times more effective in stimulating release of growth hormone than was either unacetylated somatostatin analogue or somatostatin.

D. Inhibition of Tyrosine Kinase Activity

As described above, proliferative activity of certain cells has been correlated to tyrosine kinase activity present in the cells. As described herein (Example 11), tyrosine kinase activity can be measured in cells from the human breast adenocarcinoma line MDA-MB-231. Briefly, these cells are incubated with a test compound, such as the polyunsaturated fatty acid-tyrosine kinase peptide inhibitor DHA-STKSI (DHA-SEQ ID NO: 2), then harvested and homogenized. Tyrosine kinase activity is measured by incorporation of radiolabeled phosphate into an endogenous protein substrate in the cells. In this case the endogenous protein substrate is the EGF receptor, and the phosphorylation event is an autophosphorylation event.

The effect of DHA-STKSI on EGF receptor autophosphorylation is shown in Table 3. At a concentration of 20 micrograms/ml, the underivatized peptide inhibitor, STKSI (SEQ ID NO:2), did not inhibit autophosphorylation of EGF. In contrast, DHA-STKSI (DHA-SEQ ID NO:2) inhibited phosphorylation by 50% at 1 microgram/ml and by 70% at 20 micrograms/ml. DHA present by itself at 20 micrograms/ml decreased the extent of phosphorylation by only 5%. It can be appreciated, in accordance with the present invention, that similar cellular as well as *in vitro* tyrosine kinase assays can be used to monitor activity and to test compounds for their abilities to inhibit such activity.

Table 3

Effect of DHA-STKSI on the Autophosphorylation of EGF Receptor

| Compound | Concentration (ug/ml) | Inhibition (%) |
|---|---|---|
| STKSI | 20 | 0 |
| DHA-STKSI | 1 | 50 |
| | 20 | 70 |
| DHA | 20 | 5 |

IV. Utility

Fatty acyl-peptide compositions of the invention are useful in inhibiting uncontrolled proliferation of cells, such as benign and malignant tumor cells, virally-infected cells, psoriatic cells, and the like. The use of the composition of the invention for inhibition of such cell proliferation is illustrated by experiments summarized in Table 1. Generally, peptides are selected for use in forming compositions of the invention, based on their known or experimentally determined activities in inhibiting cell proliferation. Selected peptides are then used to form polyunsaturated fatty acyl-peptide compositions, according to the general methods described in Section II, above and detailed in Examples 1-7. As illustrated herein, polyunsaturated fatty acyl-peptide compositions of the present invention are effective to produce a several-fold enhancement of anti-proliferative activity, in comparison to peptides alone.

More specifically, fatty acyl-GnRH and -GnRH analogues will find use in treating androgen-dependent prostate adenocarcinomas. Test GnRH compounds can be screened in an LH release assay, as described in Example 9, then tested in an experimental animal model, such as a rat bearing the Dunning R-3327-H prostate adenocarcinoma. Additionally, GnRH derivatives of the invention are expected to find usefulness in treatment of estrogen-dependent mammary tumors, and their efficacy can be measured in rats (such as

Wistar Furth rats) carrying a mammary tumor such as the MT/W9A mammary tumor, according to methods known in the art. Likewise, it is anticipated that such LHRH analog compositions will inhibit growth of certain pituitary tumors, chondrosarcomas, and osteosarcomas (Schally).

An anti-neoplastic or anti-tumor treatment method, as described herein, includes exposing target neoplastic cells to a concentration of fatty acyl-peptide compound effective to inhibit neoplastic cell proliferation at least about 50%, and preferably about 90%. Such effective concentrations can be determined in an *in vitro* assay, as described in Section IV, above.

Further, it can be appreciated from the foregoing discussion that the method of the invention has general utility in enhancing biological activity of a biologically active peptide, by attaching to the peptide a polyunsaturated fatty acyl moiety.

The following examples illustrate, but in no way are intended to limit, the present invention.

## Example 1

## Synthesis of Peptides

Solid-phase synthesis was carried out according to standard methods. In one exemplary method, the synthesis is performed using a Beckman model 900 peptide synthesizer. Each BOC protected amino acid (2.4 mmol) is dissolved in 5 ml dichloromethane (DCM) and cooled to 0 degrees. The volume of dichloromethane used for BOC-leucine is 12 ml, and the solution is not cooled. 2 ml of 0.6 M N,N-dicyclohexylcarbodiimide (DCCD) in DCM is added and the mixture is stirred at 0 degrees for 15 minutes. Coupling reactions are monitored at each step using the ninhydrin assay. Coupling reactions that are incomplete are repeated using the appropriate symmetric anhydride.

After the coupling reaction any remaining free amino acids are acetylated by using acetylimidazole.

Precipitation of N,N-dicyclohexylurea is completed by storage at -20 degrees for 1.5 hours, after which the precipitate is filtered and washed with ethyl ether (5 ml). The filtrate is evaporated to remove solvents, and the product is crystallized by precipitation (Meyers).

Side chain protecting groups include for cysteine, 4-Met-Benzyl; for lysine, 2-chlorobenzyloxycarbonyl; for serine, benzyl; for arginine, tosyl; for threonine, benzyl; for aspartate, benzyl; and for tyrosine, 2-bromobenzyl-oxycarbonyl. After peptide synthesis products are deprotected in liquid hydrofluoric acid. For example, a mixture of protected peptide resin (1.32 g), 2-mercaptopyridine (0.5 g), p-cresol (2.6 g), and liquid hydrogen fluoride (HF) (25 ml) is stirred at 0 degrees with a rapid stream of nitrogen gas, first below 0 degrees, then at 24 degrees. The mixture is stirred in ethyl acetate (25 ml) until a finely divided solid is obtained. The solid is filtered, washed with ethyl acetate, and air dried. The solid is stirred in 50% aqueous acetic acid (10 ml) to dissolve the peptide, filtered and washed with 20 ml water. The filtrate is freeze dried.

## Example 2

### Preparation of DHA-STKS (DHA-SEQ ID NO: 1)

A. Preparation of DHA-O-N-Succ Ester

0.340 mM DHA (112.5 mg) (Aldrich Chemical Co., catalog No.:27,155-1) and 0.341 mM TBTU (113.5 mg) were stirred in 4 ml of dry dimethylformamide (DMF) in the presence of 0.1 mM (15 mg) of diisopropylethylamine (DIEA). Activation was carried out under He atmosphere for under an hour. 0.341 mM (39.6 mg) of N-hydroxysuccinnimide (HO-N-Succ) and 0.341 mM of DIEA were added to the reaction mixture. The mixture was stirred overnight under He atmosphere for the completion of the transesterification reaction. This reaction mixture can be used directly for the N-terminal acylation of the required peptide or worked up for the

preparation of pure DHA-O-N-Succ. The reaction mixture was diluted to 30 ml with water and extracted with 3 x 10 ml of peroxide-free ether (treated and stored over alumina). The combined ether phase was back-extracted with 3 x 5 ml of water. The ether solution was dried over anhydrous sodium sulphate, evaporated to dryness and taken up in 4 ml of acetone.

B. Preparation of the N-DHA-derivative of STKS

0.03 mM STKS (37.0 mg) was first suspended in a water-acetone mixture (1:1), before DIEA (0.15 mM, 18.0 mg) was added. 0.05 mM of DHA-O-N-Succ ester in 1 ml of acetone was added to the suspension and the ratio of water-acetone was adjusted to 1:1. The reaction mixture was stirred overnight under a He atmosphere, and then evaporated to dryness. The dried residue was triturated with 3 x 5 ml of the following solvents: petroleum ether, diethyl ether, and HPLC grade water.

Using this procedure, a yield of 80% and at least a 90.0% product purity can be achieved, according to HPLC analysis. A Hewlett-Packard HP-1089 liquid chromatograph equipped with diode array detector was used with a Zorbax SB-300-C18 column with the dimensions 4.6 x 150 mm. The product's absorbance at 210 nm was monitored. The solvent composition was used with a gradient from 0 to 100% B, where buffer A consisted of 0.05% TFA in water (v/v); and buffer B consisted of 0.05% TFA in acetonitrile (v/v). The product eluted at about 64% buffer B. The chemical characterization of the compound was carried out by mass spectrometry (MS).

MH+ theoretical:1533.3,
MH+ measured:1533.2

## Example 3

## Preparation of DHA-STKSI (DHA-SEQ ID NO. 2)

A procedure similar to that described in Example 2 was used to generate the activated ester group of DHA (DHA-O-N-succinimide). Then, 0.03 mM of STKSI (36.2 mg) was acylated with 0.05 mM of DHA-O-N-Succ ester in the presence of 0.15 mM of DIEA according to Example 2. The product was eluted from the same HPLC gradient as described in Example 2, the product eluted at 64.5% B. The final yield was 82%. The chemical characterization of the compound was carried out by mass spectrometry. MH+theoretical:1517.4, MH+measured:1515.5.

Example 4

Preparation of DHA-SPKCS (DHA-SEQ ID NO: 3)

SPKC-(Lys/epsilon-TFA/)2,9 (54.8 mg, 0.04 mM, final concentration) was suspended in 3 ml of water-acetone (1:1) containing 0.08 mM DIEA. Crude DHA-O-N- Succ activated ester in DMF solution (0.04mM), as described in Example 2, was added, and the suspension was stirred overnight under a He atmosphere. The reaction mixture was evaporated to dryness and the product was TFA-deprotected in the following fashion. TFA-deprotection and purification of the end product was performed in the following fashion. The dry residue was dissolved in 3 ml of 1 M aqueous piperidine and stirred at room temperature. Deprotection was complete within 135 min. The reaction mixture was neutralized with acetic acid, evaporated to dryness and triturated with 3 x 5 ml of petroleum ether. The rest of the solvent was evaporated, the residue was taken up in 4 ml of water-glacial acetic acid (1:1) and purified by preparative HPLC. Preparative HPLC conditions were as follows. An Aquapore RP-300 column (Source) 2 x 22 cm was used with a buffer gradient of 0-100% buffer B. Buffer A was 0.05% TFA in water; buffer B was 0.05% TFA in acetonitrile. The flow rate was 10 ml/min and the eluent program used was the following:

| t(min) | A% | B% |
|---|---|---|

| | | |
|---|---|---|
| 0 | 100 | 0 |
| 10 | 95 | 5 |
| 25 | 95 | 5 |
| 45 | 50 | 50 |
| 65 | 0 | 100 |

The product was eluted at 11.5 minutes, the yield was 65%, the purity of the product was 97%. The product was analyzed by mass spectrometry, with the following results: MH+theoretical:1700.8; MH+measured:1700.8

## Example 5

## Preparation of DHA-Somatostatin Analogue (DHA-SEQ ID NO: 4)

360 ul each of 0.5 mM solutions of DHA, N,N'-diisopropyl carbodiimide (DIC), and pentafluorophenol in dimethylformamide (DMF) were mixed and kept at 25.oC. After 15 min 54 mg (45 umol) of H-D-Phe-Cys-Tyr-D-Trp--Lys(Tfa)-Val-Cys-Thr-NH2 x HCl (dissolved in 1 ml of DMF) were added to this solution and the pH of the reaction mixture was adjusted to 8 with triethylamine and kept at room temperature overnight. The DMF was evaporated *in vacuo*, and the oily residue was dissolved in 1 ml of ethanol. To the solution was added 2 ml of 2 M hydrazine hydrate in ethanol. and the reaction mixture was stirred at 45°C for 48 hours.

The solvent was removed *in vacuo* and the oily residue was TFA-deprotected by dissolving the residue in 3 ml of 1 M aqueous piperidine and stirring at room temperature, and dissolved in 28 ml of a 2-propanol:acetic acid:water (30:35:35) solvent mixture and purified by HPLC under the following conditions: A reversed-phase chromatography column was used (Prepex C-18, 25-40 um, 42 x 1.4 cm); the buffer was a 2-propanol:acetic acid:water mixture (30:35:35) and separation was followed by TLC and HPLC. THe purest fractions were pooled and repurified by MPLC. The elution procedure was the following:

Step 1: Isocratic elution with 50 ml of 2-propanol-acetic acid-water (20:40:40).

Step 2: Gradient elution with 400 ml of elution mixture applied in step 1 and 400 ml of 2-propanolacetic acid-water (35:32.5:32.5).

The purity of the fractions was checked by HPLC, the purest fractions were pooled, evaporated, lyophilized and yielded 57% product. The purity of the product was 92%. The composition of the product was analyzed by mass spectrometry.

MH+theoretical:1356.5.

MH+measured:1356.7

## Example 6

### Preparation of DHA-D-Lys6-GnRH Analogue (SEQ ID NO: 5)

360 ul each of 0.2 mM solutions of DHA, DIC, and pentafluorophenol in DMF were mixed and kept at room temperature. After 15 minutes 56 mg (0.05 mM) of Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH2 x HCl (D-Lys6-GnRH) (dissolved in 1 ml of DMF) were added to this solution and the pH of the reaction mixture was adjusted to 8 with triethylamine and kept at room temperature overnight. The product was TFA-deprotected as described in Example 4. The solvent was removed in vacuo and the oily residue was dissolved in 25 ml of 2-propanol-acetic acid-water (30:35:35) solvent mixture and purified by MPLC as described in Example 5. The final product yield was 47%. The sample identity was confirmed by mass spectrometry.

MH+theoretical:1564.9,

MH+measured:1565.0

## Example 7

### Synthesis of DHA-T Cell Epitope Peptide (DHA-SEQ ID NO: 12)

T-cell epitope peptide (TCEP) (132 mg, 0.075 mmole), with TFA and formyl protecting groups, was dissolved in DMSO (2 ml) containing DIEA (40 µl, 0.30 mmole) and DHA succinyl ester (0.075 mmole). The reaction mixture was stirred for 3 hours at room temperature at which time acetonitrile/water (1:1, v/v), 0.5 ml) and TFA (0.5 ml) were added. The reaction mixture was then filtered through a 0.2 µm filter and this solution was applied directly to a preparative reversed-phase HPLC column for purification. DHA-TCEP was collected and identified by mass spectroscopy. To remove the TFA and formyl protecting groups the purified peptide was treated in 1 M aqueous piperidine for 8 hours at room temperature at which time the product was isolated by lyophilization. The product was identified by mass spectrometry ($MW_{calc}$ = 1886.7, $MW_{found}$ = 1888.5).

## Example 8

## Assay of the Inhibition of Cell Proliferation

The growth inhibitory effect of peptide hormone analogues, such as DHA-STKS, DHA-STKCS and D-Lys6(DHA)-GnRH, was evaluated on the basis of changes in the total cell number. Cells were cultivated in RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany, Cat. No.: 074-01800) supplemented with 10% fetal calf serum. Tumor cells were washed with phosphate-buffered saline, then 0.25% trypsin was added to detach them. The cells were suspended in RPMI 1640 medium and after resuspending the cells were stained with trypan blue and counted in a Burker chamber. 0.1 ml cell suspension and 0.1 ml 0.1% trypan blue were mixed. The viability of tumor cells was estimated on the basis of trypan blue (0.4%) exclusion using a hematocytometer.

## Example 9

## Luteinizing Hormone (LH) Release Assay

Anterior pituitaries were obtained from adult (male or female) Wistar strain rats according to the method

previously described (Shaw). The pituitaries were cut into small pieces, incubated with collagenase then dispersed mechanically to single cells. The cell-suspension was mixed with Sephadex G-10 beads as support material and transferred into a superfusion chamber. The cells were continuously perfused with oxygenated medium or with a medium containing the peptide to be tested, such as D-Lys6(DHA)-GnRH. The LH content from each 1 ml fraction of the superfusate was measured by radioimmunoassay (RIA) using rat an LH RIA kit or as described in Example 9 using radiolabeled LH and anti-luteinizing hormone antibodies. The biological potency of a given analogue was determined based on the pituitary hormone responses (peaks) to the peptide stimulation over the baseline secretion.

Example 10

Assay for Growth Hormone (GH)

The release of GH was measured during various DHA-peptide hormone or peptide analogue treatments, such as somatotsatin analogue treatments, on rat hypophysis by using the superfusion method (Myers). Following is a description of the method. The hypophyses are cut into small pieces, incubated with collagenase, and dispersed to single cells. The cells in a superfusion chamber are continuously perfused with oxygenated medium or with medium containing the peptide to be tested.

The levels of GH are determined by a double-antibody radioimmunoassay for the hormone. The following methodology can be used. Several commercial sources now supply reagent grade hGH of sufficient purity to be reliably used in the assay. Antibody is produced by dissolving an appropriate amount of growth hormone (2-3 mg) in 200 microliters 0.01 N sodium hydroxide. The solution is diluted to 1 mg/ml with protein free PBS and mixed 1:1 with Freund's adjuvant. 0.5-1 mg growth hormone is injected into three subcutaneous sites of a young guinea pig,

and repeated at two- to three-week intervals. The anti-hGH serum is harvested from the guinea pigs and tested for its immunopotency. Antisera which will allow for a sensitive assay usually will precipitate 50% of the labelled hGH at a final dilution of 1:50,000 using test radiolabelled hGH. Second antibodies (goat anti-guinea pig antibodies) are prepared in a similar fashion. The following radioiodination procedure for hGH is used. One ampoule containing hGH in 20 microliters is thawed and placed on ice. I-125 is usually obtained from New England Nuclear (Boston, MA). High specific activity exceeding 200 mCi/ml is necessary for the highest degree of sensitivity in the assay. 0.5 M phosphate buffer (pH 7.6) is added to the ampoule containing hGH. About 1 mCi of the radioactive iodine is added to the ampoule.

To start the iodination reaction, 35 micrograms of a freshly prepared solution of chloramine-T in 25 microliters 0.05 M phosphate buffer (pH 7.6) is added. The iodine, hormone, and oxidant are gently agitated for 15 seconds. To stop the reaction, 125 micrograms of a freshly prepared solution of sodium metabisulfate in 100 microliters of 0.05 M phosphate (pH 7.6) is added. The entire contents of the ampoule are then placed on Sephadex G-50 column and the labeled hormone separated from the iodine. The hormone is diluted in 1% BSA-PBS and stored in small aliquots at -20°C.

The hGH radioimmunoassay is performed in the following manner. The standard's concentrations in hGH assays are the following: 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 ng/ml. 50 ml of the unknown growth hormone concentration is added to tubes not containing the standard. The next step is to add the guinea pig anti-hGH serum (first antibody). The radiolabeled hGH is added to every tube. The assay tubes are shaken gently, left at room temperature for two hours, and then placed in a refrigerator at 4°C for at least three days. After the three-day incubation, the

tubes are removed from the refrigerator and the appropriate dilution of the goat anti-guinea pig serum added (second antibody). The second antibody is diluted in 1% BSA PBS buffer to obtain approximately 50% precipitation of antibody-bound labeled hGH. Aliquots of 100 microliters of the second antibody are added to every tube. The tubes are gently agitated, left at room temperature for about two hours, and then placed at 4°C. for at least eight hours.

Following incubation with the second antibody, the tubes are centrifuged at 2000 g for 30 minutes at 4 degrees in a refrigerated IEC-PR6 centrifuge. The supernatants are aspirated by vacuum suction, with care taken not to disturb the precipitates packed in the bottom of tubes. The pellet is resuspended in 2 ml cold PBS and recentrifuged as described above. Washing the precipitates with buffer increases the reproducibility.

## Example 11

## Measurement of Autophosphorylation of the EGF Receptor

Autophosphorylation of the EGF receptor was measured according to Bellot *et al.* (Bellot). Cells were washed once with binding buffer. The plates were then placed in a water bath at 37 degrees and different concentrations of the analogues were added to the cells for specific periods of time. The buffer was removed and cells were scraped off the plates with 0.5 ml of Laemmli's sample buffer, boiled for 5 minutes, and sonicated for 10 seconds. Aliquots of each sample were run on two different SDS-polyacrylamide gels (7%) and each gel was transferred to nitrocellulose paper. One was immunoblotted with RK2, an anti-EGF-R antibody, and the other with an antiphosphotyrosine-specific polyclonal antibody. Blots were then labeled with radioiodinated Protein A (New England Nuclear) and autoradiograms of the nitrocellulose papers were made on Kodak X-Omat paper. The activity of the analogues was characterized on the basis of their inhibitory effect on

the phosphotyrosine content in comparison to that of untreated cells used as control. Alternatively, the tyrosine kinase activity was determined according to the following method (Keri). The cells were incubated with the substrate analogues, such as DHA-SPRCS or DHA-STRS for 24 hours, then harvested and homogenized. The reaction volume of 100 microliters, and homogenized in a Dounce homogenizer 30 times in 5 vol buffer (50 mM Tris-HCl, pH 7.8, 50 mM MgCl2, 10 micromolar sodiumvanadate, 1 mM EDTA, and 50 microgram/ml aprotinin. The reaction volume of 100 ml contained 50 mM Tris-HCl, pH 7.8, 50 mM MgCl2, 10 micromolar sodium vanadate, 0.1% nonidet P-40, 5 micromole gamma 32p-ATP, 1mM substrate and 60 microliter homogenate. The assay was initiated by addition of the ATP.

After incubation the reaction was stopped by addition of trichloroacetic acid, and the supernatant was spotted on a 2 x 2 cm phosphocellulose paper (Whatman P-81). The paper squares were washed with phosphoric acid and acetone, and the dried papers were counted for radioactivity in scintillation fluid. For each sample an appropriate reaction mixture containing no peptide was run a control. The activity of the analogues was characterized on the basis of their inhibitory effect in comparison to the incorporation of 32P isotope to untreated cells used as control.

## Example 12

## Preparation of DHA-EGFA (SEQ. ID. NO: 6)

0.035 mM of EGFA was suspended in 2.5 ml of water-acetone (1:1), then 0.035 mM DIEA (6.5 ul) and 0.035 mM of DHA-O-N-Succ ester (40.2 mg) in 2.0ml DMF were added to the solution. The acylation was carried out by stirring the reaction mixture overnight under He atmosphere. The solvents were evaporated and the dry residue was triturated with 3 x 5 ml of petroleum ether. The solvent residue was

removed and the crude product was purified by preparative HPLC under the following circumstances:

Eluent programme:

| t(min) | A% | B% |
|---|---|---|
| 0 | 100 | 0 |
| 10 | 95 | 5 |
| 25 | 95 | 5 |
| 45 | 40 | 60 |
| 55 | 40 | 60 |
| 65 | 0 | 100 |

All the other parameters were identical to those in Example 3.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

1. A fatty acyl-peptide composition effective to inhibit cell proliferation comprising

a peptide having antiproliferative activity, and

a polyunsaturated fatty acyl moiety, which is conjugated to the peptide through the acyl group of said moiety,

said composition being characterized by a cell proliferative inhibitory activity which is several-fold greater than that of the peptide alone.

2. The fatty acyl-peptide composition of claim 1, wherein the polyunsaturated fatty acyl moiety is a docosahexaenoyl or an eicosapentaenoyl moiety.

3. The fatty acyl-peptide composition of claim 2, wherein the polyunsaturated fatty acyl moiety is selected from the group consisting of cis-4,7,10,13,16,19-docosahexaenoyl (DHA) and cis-5,8,11,14,17-eicosapentaenoyl (EPA) moieties.

4. The fatty acyl-peptide composition of claim 1, wherein said peptide is a peptide hormone.

5. The fatty acyl-peptide composition of claim 4, wherein the peptide hormone is selected from the group consisting of somatostatin analogs and GnRH analogs.

6. The fatty acyl-peptide composition of claim 4, wherein the peptide hormone is SEQ ID NO: 4.

7. The fatty acyl-peptide composition of claim 4, wherein the peptide hormone is SEQ ID NO: 5.

8. The fatty acyl-peptide composition of claim 1, wherein said peptide is a protein kinase modulatory peptide.

9. The fatty acyl-peptide composition of claim 8, wherein the peptide is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 6.

10. The fatty acyl-peptide composition of claim 1, wherein the polyunsaturated fatty acyl moiety is conjugated to the peptide through an amide linkage.

11. A method for producing a several-fold enhancement of cell proliferative inhibitory activity in a peptide composition comprising

conjugating the peptide with a polyunsaturated fatty acid to form a fatty acyl-peptide composition composed of the peptide and a fatty acyl moiety conjugated to the peptide through the acyl group of said moiety.

12. The method of claim 11, wherein the polyunsaturated fatty acyl moiety is a docosahexaenoyl or an eicosapentaenoyl moiety.

13. The method of claim 12, wherein the polyunsaturated fatty acyl moiety is selected from the group consisting of cis-4,7,10,13,16,19-docosahexaenoyl (DHA) and cis-5,8,11,14,17-eicosapentaenoyl (EPA) moieties.

14. The method of claim 11, wherein the polyunsaturated fatty acyl moiety is conjugated to the peptide through an amide linkage.

15. The method of claim 11, wherein said peptide is a peptide hormone.

16. The method of claim 15, wherein the peptide hormone is selected from the group consisting of somatostatin analogs and GnRH analogs.

17. The method of claim 15, wherein the peptide hormone is SEQ ID NO: 4.

18. The method of claim 15, wherein the peptide hormone is SEQ ID NO: 5.

19. The method of claim 11, wherein said peptide is a protein kinase modulatory peptide.

20. The method of claim 19, wherein the peptide is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 6.

| SEQ ID NO. | SEQUENCE |
|---|---|
| 1 | E D A E Y A R R G |
| 2 | E D A E F A R R G |
| 3 | Q K R P S Q R S K Y L |
| 4 | dF C Y dW K V C T $NH_2$ |
| 5 | Glp H W S Y dK L R P G $NH_2$ |
| 6 | C H S G Y V G V R C |
| 7 | S V S E I Q L M H N L G K H L N S M E R V E W L R K K L Q D V H N F |
| 8 | G R G D S P A |
| 9 | V W K G L P R E Y V S D K |
| 10 | R R L I E D N E Y T A R G |
| 11 | R R L I E Y A A R G |
| 12 | C(ACM) M H I E S L D S Y L I P T C C(ACM) |
| 13 | R A G G Y R F Y D Y R K |
| 14 | T A E N A E Y L R V A P |
| 15 | E D A E Y A R R R G |
| 16 | P G E Y N Y N D N P M E E E E |
| 17 | L S E F S F K K |
| 18 | R R K A S G P |
| 19 | L R R A S L G |
| 21 | L S Y R R Y S L |
| 20 | L S Y R R Y S L |
| 22 | N Y L R R L S D S N F |
| 23 | T R N T S V S G N A P P K |
| 24 | S E L S R R |
| 25 | A K A K K T P K K |
| 26 | R P P G F S F F R |
| 27 | P K T P S G E A |
| 28 | S D S E E E N D |
| 29 | S S E E E |
| 30 | M R R S V S E A A L |
| 31 | R R L S S F K K |

Fig. 1

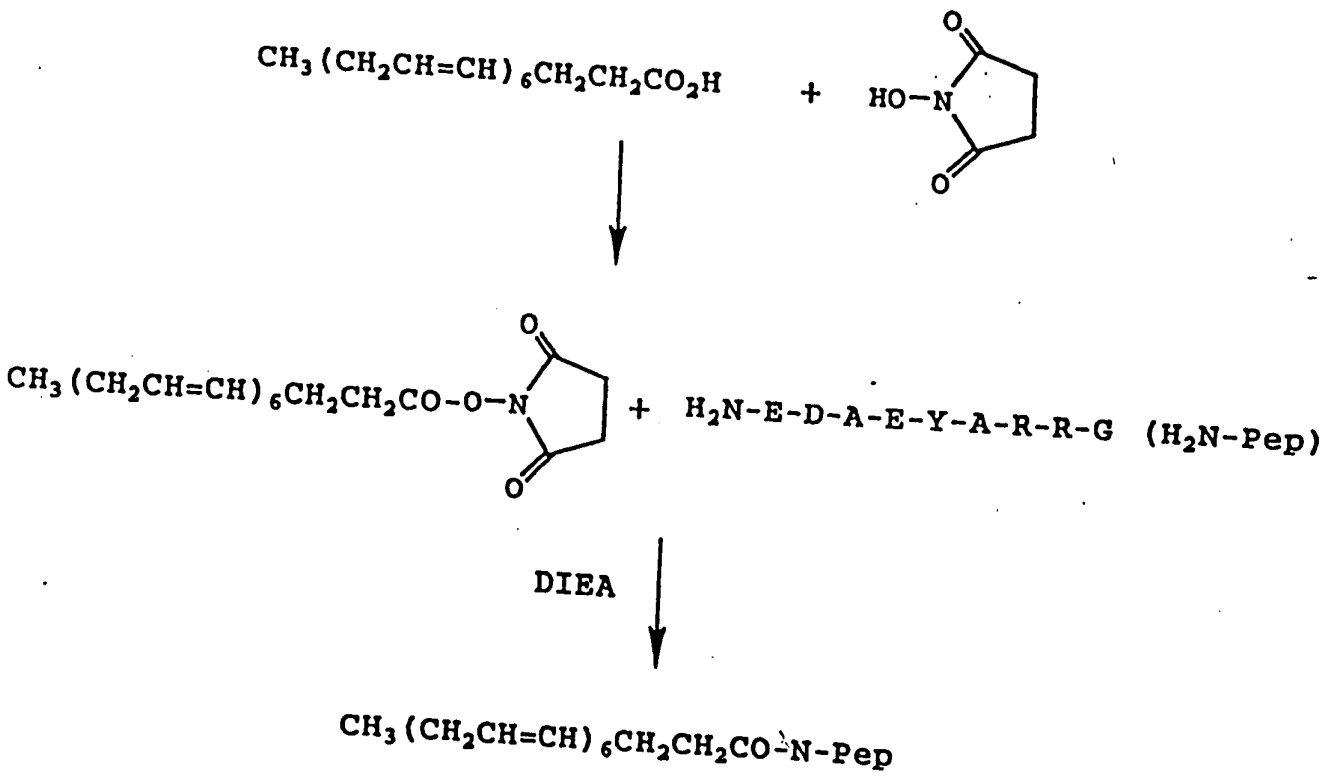
CH3(CH2CH=CH)6CH2CH2CO2H
+
HO-N
O
O
CH3(CH2CH=CH)6CH2CH2CO-O-N
O
O
+ H2N-E-D-A-E-Y-A-R-R-G (H2N-Pep)
DIEA
CH3(CH2CH=CH)6CH2CH2CO-N-Pep

Fig. 2

| COMPOSITION | STRUCTURE |
|---|---|
| I | DHA-E D A E Y A R R G |
| II | DHA-E D A E F A R R G |
| III | DHA-Q K R P S Q R S K Y L |
| IV | DHA-dF C Y dW K V C T $NH_2$ |
| V | DHA-Glp H W S Y dK L R P G $NH_2$ |
| VI | DHA-C H S G Y V G V R C |

Fig. 3

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/HU 93/00065

A. CLASSIFICATION OF SUBJECT MATTER

IPC[5]: C 07 K 7/06, 7/08; A 61 K 37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[5]: C 07 K; A 61 K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Chemical Abstracts, Volume 111, No. 15, issued 09 October 1989 (09.10.89), Columbus, Ohio, USA, A.V. Kabanov et al. "Fatty acid acylated antibodies against virus suppress its reproduction in cells", page 580, left column, abstract No. 132 137 v & FEBS Lett. 1989, 250(2), 238-40 | 1 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 March 1994 (17.03.94) | 21 April 1994 (21.04.94) |

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